Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Titration of Feline Rhinotracheitis Virus in Cell Culture

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes an *in vitro* titration method for assaying modified-live feline rhinotracheitis virus (FRV) vaccines for viral content. The method uses plaque-forming units (PFU) in a cell culture system for titration of FRV.

1.2 Keywords

Feline rhinotracheitis virus, FRV, PFU, potency test, titration, *in vitro*, plaque

2. Materials

2.1 Equipment/instrumentation

2.1.1 Syringe,[1] self-refilling, repetitive, 2 ml

2.1.2 Micropipettor,[2] 200 µl, and tips[3]

2.1.3 Blender[4]

2.1.4 Media bottle,[5] borosilicate glass with screw-top lid, 1000 ml

2.1.5 Incubator,[6] 36° ± 2°C, high-humidity, 5% ± 1% CO_2

2.1.6 Water bath[7]

2.1.7 Inverted light microscope[8]

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[1] Wheaton® 13-689-50C, Fisher Scientific Corp., 2000 Park Ln., Pittsburg, PA 15275 or equivalent

[2] Pipetman® Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent

[3] Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Center, NY 11571 or equivalent

[4] Waring blender, Cat. No. 14-509-35, Fisher Scientific Corp. or equivalent

[5] Wheaton® 219760, Fisher Scientific Corp. or equivalent

[6] Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent

[7] Cat. No. 15-461-10, Fisher Scientific Corp. or equivalent

[8] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent

2.1.8 Vortex mixer[9]

2.1.9 Pipette-aid[10]

2.2 Reagents/supplies

2.2.1 FRV Reference,[11] C-27 strain

2.2.2 Crandell feline kidney (CRFK) cell culture,[12] free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR).

2.2.3 Minimum essential medium (MEM)

2.2.3.1 9.61 g MEM with Earle's salts without bicarbonate[13]

2.2.3.2 2.2 g sodium bicarbonate ($NaHCO_3$)[14]

2.2.3.3 Dissolve with 900 ml deionized water (DW).

2.2.3.4 Add 5.0 g lactalbumin hydrolysate or edamine[15] to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to **Section 2.2.3.3** with constant mixing.

2.2.3.5 Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[16]

2.2.3.6 Sterilize through a 0.22-µm filter.[17]

2.2.3.7 Aseptically add:

1. 25 units/ml penicillin[18]

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[9] Model G-560, Scientific Industries, Inc., 700 Orville Dr., Bohemia, NY 11716 or equivalent

[10] Cat. No. 183, Drummond Scientific Co., 500 Pkwy., Broomall, PA 19008 or equivalent

[11] Reference quantities available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent

[12] CCL-94, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776

[13] Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent

[14] Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

[15] Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

[16] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

[17] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

[18] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent

2. 50 µg/ml gentamicin sulfate[19]

3. 100 µg/ml streptomycin[20]

2.2.3.8 Store at 4° ± 2°C.

2.2.4 Growth Medium

2.2.4.1 900 ml of MEM

2.2.4.2 Aseptically add:

1. 100 ml gamma-irradiated fetal bovine serum (FBS)

2. 10 ml L-glutamine[21]

2.2.4.3 Store at 4° ± 2°C.

2.2.5 2X Medium

2.2.5.1 19.22 g MEM with Earle's salts without bicarbonate

2.2.5.2 2.2 g $NaHCO_3$

2.2.5.3 Dissolve with 900 ml DW.

2.2.5.4 Add 5.0 g lactalbumin hydrolysate or edamine to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to **Section 2.2.5.3** with constant mixing.

2.2.5.5 Q.S. to 1000 ml with DW, and adjust pH to 6.8-6.9 with 2N HCl.

2.2.5.6 Sterilize through a 0.22-µm filter.

2.2.5.7 Store at 4° ± 2°C.

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[19]Gentocin solution, Cat. No. 0061-0464-04, Schering Laboratories or equivalent
[20]Cat. No. S-9137, Sigma Chemical Co. or equivalent
[21]L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent

2.2.5.8 Prior to preparing the Overlay Medium (**Section 3.3.3**), aseptically add:

1. 100 units/ml penicillin

2. 50 µg/ml gentamicin sulfate

3. 100 µg/ml streptomycin

2.2.6 2% Tragacanth Gum (Trag)

2.2.6.1 20 g Trag[22]

2.2.6.2 1000 ml DW

2.2.6.3 Mix small amounts of Trag at a time vigorously, with a blender set on high.

2.2.6.4 Pour 500 ml each into 1000-ml media bottles.

2.2.6.5 Sterilize by autoclaving at 15 psi, 121° ± 2°C for 35 ± 5 min.

2.2.6.6 Store at 4° ± 2°C.

2.2.7 7.5% Sodium Bicarbonate

2.2.7.1 7.5 g $NaHCO_3$

2.2.7.2 Q.S. to 100 ml with DW.

2.2.7.3 Sterilize by autoclaving at 15 psi, 121° ± 2°C for 30 ± 10 min.

2.2.7.4 Store at 4° ± 2°C.

2.2.8 70% Ethyl Alcohol

2.2.8.1 73 ml ethyl alcohol[23]

2.2.8.2 27 ml DW

[22]Acros AC42138-5000, Fisher Scientific Corp. or equivalent
[23]Denatured, 190 proof, Cat. No. 7018, J.T. Baker, Inc. or equivalent

2.2.8.3 Store at room temperature (RT) (23° ± 2°C).

2.2.9 Crystal Violet Stain

2.2.9.1 7.5 g crystal violet[24]

2.2.9.2 50 ml 70% Ethyl Alcohol

2.2.9.3 Dissolve crystal violet in alcohol; add remaining ingredients.

2.2.9.4 250 ml formaldehyde[25]

2.2.9.5 Q.S. to 1000 ml with DW.

2.2.9.6 Filter through a Whatman® #1 filter.[26]

2.2.9.7 Store at RT.

2.2.10 Tissue culture plates,[27] 4 well

2.2.11 Polystyrene tubes,[28] 12 x 75 mm

2.2.12 Pipettes,[29] 25 ml

2.2.13 Needles,[30] 18 ga x 1½ in

2.2.14 Syringes,[31] (tuberculin) 1 ml

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[24]Cat. No. C 0775, Sigma Chemical Co. or equivalent
[25]37% by weight, Cat. No. F79, Fisher Scientific Corp. or equivalent
[26]Cat. No. 1001, Fisher Scientific Corp. or equivalent
[27]Cat. No. 7603705, ICN Biochemicals, Inc., 3300 Hyland Ave., Costa Mesa, CA 92626 or equivalent
[28]Falcon® 2058, Becton Dickinson Labware, 1 Becton Dr., Franklin Lakes, NJ 07417 or equivalent
[29]Cat. No. 13-675-30, Fisher Scientific Corp. or equivalent
[30]Cat. No. 305196, Becton Dickinson Labware or equivalent
[31]Cat. No. 309602, Becton Dickinson Labware or equivalent

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in the preparation and maintenance of cell culture as well as in the propagation and maintenance of animal viruses and the quantification of virus infectivity by plaque formation.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set a water bath at 36° ± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of CRFK tissue culture plates (Test Plates)

Cells are prepared from healthy, confluent CRFK cell cultures that are maintained by passing every 3 to 4 days. Two days prior to test initiation, add 8.0 ml/well of approximately $10^{5.0}$ to $10^{5.3}$ cells/ml suspended in Growth Medium into all wells of the 4-well tissue culture plate. Prepare 1 Test Plate for the FRV Reference Control and 1 for each Test Serial. Incubate at 36° ± 2°C in a CO_2 incubator.

3.3.2 Preparation of FRV Reference Control

3.3.2.1 On the day of test initiation, rapidly thaw a vial of FRV Reference in the water bath.

3.3.2.2 Using a self-refilling, repetitive syringe, dispense 1.8 ml of MEM into sufficient 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the CVB-L Reference and Reagent sheet; appropriately label (e.g., 8 tubes, labeled 10^{-1} through 10^{-8}, respectively).

3.3.2.3 Transfer 200 µl of the FRV Reference to the tube labeled 10^{-1}; mix by vortexing.

3.3.2.4 Using a new pipette tip, transfer 200 µl from the 10^{-1}-labeled tube to the 10^{-2} tube; mix by vortexing.

3.3.2.5 Repeat **Section 3.3.2.4** for each of the subsequent dilutions, transferring 200 µl of the previous dilution to the next dilution tube until the tenfold dilution series is completed.

3.3.3 On the day of test initiation, prepare Overlay Medium. Approximately 35 ml per plate is required.

3.3.3.1 Aseptically add to 1 volume of 2X Medium:

1. 10% gamma-irradiated FBS
2. 2% 7.5% Sodium Bicarbonate
3. 1 volume of 2% Trag

3.3.3.2 Mix and warm the Overlay Medium in the water bath for 60 ± 10 min prior to performing the procedure in **Section 4.5**.

3.4 Preparation of the Test Serial

3.4.1 The initial test will be with a single vial (a single sample from 1 vial). On the day of test initiation, using a 1.0-ml syringe and an 18-ga x 1½-in needle, rehydrate a vial of the Test Serial with the provided diluent by transferring 1.0 ml for a 1-ml-dose vaccine, 0.5 ml for a ½-ml-dose vaccine, etc., into the vial containing the lyophilized Test Serial; mix by vortexing. Incubate for 15 ± 5 min at RT.

3.4.2 Dispense 1.8 ml MEM into each of 6, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-6} respectively, using a 2-ml self-refilling, repetitive syringe.

3.4.3 Transfer 200 µl of the Test Serial to the tube labeled 10^{-1}; mix by vortexing.

3.4.4 Using a new pipette tip, transfer 200 µl from the tube labeled 10^{-1} to the 10^{-2} tube; mix by vortexing.

3.4.5 Repeat **Section 3.4.4** for each of the subsequent dilutions, transferring 200 µl of the previous dilution to the next dilution tube until the tenfold dilution series is completed.

4. Performance of the test

4.1 On the day of test initiation, label the Test Plates and remove the Growth Medium.

4.2 Inoculate 1 well/dilution with 200 µl from dilutions 10^{-6} through 10^{-3} of the Test Serial. Inoculate 1 well/dilution from dilutions 10^{-8} through 10^{-6} of the FRV Reference Control. Change tips between each unique sample (i.e., each Test Serial), but tip changes are not necessary between each dilution in a series when pipetting from the most dilute to the most concentrated (e.g., 10^{-8} through 10^{-6}). Gently rotate the plates to evenly disperse the inoculum.

4.3 One uninoculated well on a Test Plate serves as a negative cell control.

4.4 Incubate the inoculated Test Plates at 36° ± 2°C for 75 ± 15 min in a CO_2 incubator. To evenly disperse the inoculum, gently rotate the plates at intervals of 25 ± 5 min.

4.5 Add 8 ml/well of the Overlay Medium to the plates with a 25-ml pipette. Discard any unused, warmed Overlay Medium.

4.6 Incubate the Test Plates undisturbed at 36° ± 2°C in a CO_2 incubator for 96 ± 12 hr.

4.7 After incubation, without removing the Overlay Medium, pipette 5 ml of the Crystal Violet Stain into each well of the Test Plates with a 25-ml pipette; mix by gentle rotation.

4.8 Allow Test Plates to incubate at RT for 25 ± 5 min.

4.9 Wash the Overlay Medium and the Crystal Violet Stain from the cell monolayers by dipping each plate several times in a container overflowing with running water from the cold water tap until the water is clear. Allow to air dry.

4.10 Count the number of FRV plaques for each well. If FRV and feline calicivirus (FCV) plaques are counted together in a combination vaccine, the FRV plaques will contrast markedly from FCV. The FCV plaques are large, clear circular areas (averaging 3 to 4 mm in diameter) with fuzzy edges. The FRV plaques are visible as small, clear plaques approximately 1 mm diameter with distinct edges. If the plaques are not distinuishable by size, use an inverted light microscope with 100X magnification to differentiate the plaque edges.

4.11 Record results as the number of FRV plaques for each dilution of a Test Serial and the FRV Reference Control.

4.12 Calculate the FRV titers of the Test Serial and the FRV Reference Control from the dilutions with plaque counts containing 10-100 plaques. The titer may be expressed as PFU per dose of vaccine.

Example:

Log_{10} of PFU (65)	= 1.8
Log_{10} of reciprocal of dilution counted (10^{-3})	= 3.0
Log_{10} of reciprocal of dose factor	
$\frac{200\ \mu l\ inoculum}{1\text{-ml dose}} = \frac{1}{5}$	$= 0.7$
Total	= 5.5

Titer of the Test Serial is $10^{5.5}$ PFU.

4.13 One plaque represents a single infective unit (IU), whereas 1, 50% tissue culture infective dose ($TCID_{50}$) is statistically equivalent to a theoretical 0.69 IU. A $TCID_{50}$ endpoint will be 1.44 times those expressed as PFU per unit of inoculation. Therefore, to express PFU titer as $TCID_{50}$ titer, multiply the PFU titer by 1.44 or add 0.16 (the log of 1.44) to the log_{10} value of the PFU titer. From the above example the titer of the Test Serial is $10^{5.66}$ $TCID_{50}$.

5. Interpretation of the test results

5.1 Validity requirements

5.1.1 The calculated titer of the FRV Reference Control must fall within plus or minus 2 standard deviations (± 2 sd) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The uninoculated cell control cannot exhibit any plaques, cytopathic effect (CPE), or cloudy media that would indicate any contamination.

5.1.3 The Test Serial and the FRV Reference Control must have dilutions in which 10 to 100 PFU are counted.

5.2 If the validity requirements are not met, then the assay is considered a **NO TEST** and can be retested without prejudice.

5.3 If the validity requirements are met and the titer of the Test Serial is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production (OP) for the product under test, the Test Serial is considered **SATISFACTORY**.

5.4 In a valid test, if the titer of the Test Serial is less than the titer contained in the APHIS filed OP for the product under test, the Test Serial may be retested in accordance with the Code of Federal Regulations, Title 9, Part 113.8(b).

6. Report of test results

Results are reported as PFU or $TCID_{50}$ per dose of Test Serial.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.315, U.S. Government Printing Office, Washington, DC, 2000.

7.2 Cottral, GE, *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates. Ithaca, NY, 1978, pg. 731.

7.3 Davis, Dulbecco, Eisen, and Ginsberg, ed., *Microbiology including Immunology and Molecular Genetics,* 3rd ed. Harper and Row, Hagertown, MD, 1980, pg 880.

8. Summary of revisions

Supplemental Assay Method for the Titration of Feline Rhinotracheitis Virus in Cell Culture

8.1 MVSAM0307.01:

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.

8.2 MVSAM0307.02:

The following is a list of the changes made from the previous protocol:

1. Changes have been made to the 2X Medium and Overlay Medium formulations.

2. 7.5% Sodium Bicarbonate has been included for preparation of the Overlay Medium.

3. A method has been added to convert the data from PFU to $TCID_{50}$.

4. Minor changes in terminology have been made and additional details have been added for clarification and consistency with other SAMs.